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COMPLETE SPECIFICATION

Pituitary Gonadotropic Hormone preparation and method for its production

We, INSTITUTO FARMACOLOGICO SERONO S.p.A., of Via Casilina 125, Rome, Italy, a Company organised under the laws of Italy, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a pituitary gonadotropic hormone preparation and a method for its production.

The different preparations with gonadotropic activity on the market are the result of research which leads to the conclusion that the human and higher animals' hypophysis elaborate and secrete two gonadotropic factors, with distinct activity. One of them is called FSH (Follicle stimulating hormone) and the other "ICSH or LH factor" (Interstitial cell stimulating hormone or luteinizing hormone).

Four different types of products with gonadotropic activity are at present available; they are:—

(1) The gonadotropic chorionic hormone, as extracted from the urine of pregnant women, and which biologically corresponds to the LH factor.

(2) The serum gonadotropic hormone, as extracted from pregnant mare serum, the activity of which corresponds to both the FHS and LH factors.

(3) The pituitary gonadotropic hormone, as extracted from the hypophysis of animals like sheep, horses and pigs, whose activity reveals itself in both FHS or LH sense.

(4) The gonadotropic hormones as extracted from the urine of women in the menopausal stage, whose biological activity corresponds to both the FHS and LH factors together. The process of extraction and purification of this hormone, which from now on will be referred to as HMG (human menopausal gonadotropin) is disclosed in British Patent Specification 903,346.

[*Price 4s. 6d.*]

All of the above-mentioned products with gonadotropic activity present rather serious inconveniences. In fact, the biological effect of the chorionic gonadotropic hormone (HCG or human chlorionic gonadotropin) is exclusively that of the LH type and therefore such a hormone is evidently only useful for the treatment of certain affliction for which the FSH type activity is unnecessary. On the other hand, it is known that both the serum or pituitary gonadotropins, i.e. those which are not of human origin, have the capacity of provoking, after repeated injections of humans during a certain period of time, the formation of specific anti-bodies or anti-hormones capable of inhibiting the action of successive administrations of the same hormone.

The gonadotropic hormone, as extracted from post-menopausal urine and which is the subject of British Patent Specification No. 903,346, in spite of its beneficial therapeutic effect, also contains impurities which limit its therapeutic usefulness in those cases in which considerable hormone dosages are required. The said HMG is so impure that it certainly cannot be utilized as an eventual model of chemical structure for the study of a synthesis of the molecule of the pituitary gonadotropic hormone, or hormones. For the same reason (that is because of the excessive quantity of impurities), the said HMG cannot be used for the eventual immunological study of gonadotropins, which aims at clarifying the problems concerning the qualitative and quantitative determination of the FHS and LH factors in human liquids (i.e. plasma, serum, urine, etc.).

The primary object of the present invention is to provide a procedure which, starting from an easily available material, allows the obtention of a human urinary gonadotropic hypophyseal hormone with predominant FSH activity and with LH activity, devoid of antigen properties when injected into humans, capable of stimulating the human ovaries and

of promoting ovulation and pregnancy in cases of primary or secondary amenorrhoea of long duration caused by insufficient pituitary gonadotropins.

Such a preparation, thanks to its high level of purity, can also be used for studying the structure of the molecule or molecules constituting human ganodatropic hormones. This study could lead to the possibility of a complete synthesis or of the transformation of certain peptic chains of animal pituitary gonadotropins for the purpose of making the latter similar, or structurally equivalent, to human pituitary gonadotropins.

According to the present invention we provide a pituitary human menopausal gonadotropic (HMG) hormone preparation comprising follicular stimulating hormone (FSH) and luteinizing hormone (LH) having a FSH:LH ratio as hereinafter defined of at least 3:1 and having a total gonadotropic activity, when determined by the mouse uterus test as hereinafter defined in excess of 80 mg equivalent International Reference Preparation (IRP) per mg.

The FSH:LH ratio is the ratio between the biological potencies of FSH and LH. The "mouse uterus test" is a non-specific method of assaying the gonadotropic activity and which measures the combined FSH and LH activities. The method is based on the increase of the uterus weight of immature female mice (21 days old) following the administration of gonadotropins.

Preferably the said FSH:LH ratio is between 3:1 and 6:1.

The pituitary HMG hormone preparation of this invention may be in the form of a pharmaceutical preparation containing as active ingredient the said pituitary HMG preparation and lactose as an excipient therefor.

A method for the production of the said pituitary gonadotropic hormone comprises acidifying menopausal or post-menopausal human urine to a pH between 4.0 and 4.5, adding thereto about 0.5% by weight of siliceous or diatomaceous earth, e.g. "Celite" —Trade Mark—and about 1% by weight kaolin (which is added as aqueous suspension), agitating this suspension for about 1 hour, separating the said siliceous or diatomaceous earth and kaolin by settling, discarding the supernatant liquid, centrifuging the precipitate, washing the centrifugate twice with acidified water at a pH of about 4.5, eluting the precipitate twice with 1M aqueous ammonia so as to obtain an eluate of a pH of between 11.0 and 11.3, mixing the eluates, adjusting the pH of the eluate to 8.5, separating the formed precipitate, acidifying the clear liquid to a pH of 5.0 to 5.5; adding thereto about 2 volumes acetone, maintaining overnight so as to obtain a precipitate, washing the precipitate with acetone, ethanol and diethyl ether, drying the residue under reduced pressure over a drying agent, taking up the precipitate in 70% aqueous ethanol containing 10% ammonium acetate, adding to the extract absolute ethanol containing 10% ammonium acetate so as to obtain a precipitate, dissolving the precipitate in 0.05M phosphate buffer of pH 7, stirring the solution with diethyl-aminoethyl-cellulose, separating and acidifying the purified liquid to pH 5.4, passing the liquid through a synthetic alumino-silicate gel cation exchanger, column e.g. a "Permutit"—Trade Mark—column, equilibrated with 0.05M acetate buffer of pH 5.4, eluting the column with 40% aqueous ethanol, containing 10% of ammonium acetate, precipitating with 95% ethanol, washing the precipitate with ethanol and ether, and optionally dissolving the precipitate in water and passing the solution through a column packed with a cross-linked dextran gel with random ether linkages to the glucose residues in the polysaccharide chains, e.g. a column packed with "Sephadex" (Trade Mark) G 200 as sold by Pharmacia, Upsala, Sweden, and collecting the first fractions of effluent.

The method of this invention may be used to purify the preparation procedure disclosed in the said British Specification 903,346, as well as to define with precision the sequence of operations used for the extraction and purification of human menopausal gonadotropin (HMG), that is a process which can be used for the preparation of pituitary gonadotropic hormones extracted from menopausal and post-menopausal urine. It is advantageous with the method of this invention that the menopausal or post-menopausal urine, after being acidified for instance with glacial acetaic acid to adjust the pH between 4.0 and 4.5, is stirred with a watery suspension of prewashed kaolin and "Celite".

In a preferred procedure the prewashed kaolin plus "Celite" are separated off, washed with acidified water at pH 4.5 with acetic acid, extracted with aqueous ammonia solution at a pH between 11 and 11.3 inclusive, centrifuged, the pH brought again to 8.3—8.7 by means of acetic acid; the precipitate therein formed is separated, acetic acid is again added until a pH between 5 and 5.5 is reached, a precipitate is then obtained with acetone, filtered off and dried. The raw precipitate obtained is washed with 95% ethanol and with ether, extracted with 70% ethanol containing 10% ammonium acetate and the clear extract thereby obtained is precipitated with absolute ethanol containing 10% ammonium acetate. The precipitate is then washed with 95% ethanol and with water.

The washed precipitate is then dissolved with a 0.05M phosphate buffer at pH 7, stirred with diethylamino-ethyl-cellulose, and the clear filtrate acidified to pH 5.4 with e.g. glacial acetic acid, is then passed through a "Permutit" column previously treated with

1N ammonia and then with 1N acetic acid, then water and finally equilibrated with 0.05M acetate buffer at pH 5.4. After adsorption of HMG on "Permutit", this is washed with 0.05M acetate buffer at pH 5.4 until the optical density of the effluent, E280 mμ, is near zero, and then the HMG may be eluted with 40% ethanol containing 10% ammonium acetate. The eluted alcohol product is precipitated with 95% ethanol, centrifuged, washed with absolute ethanol, and then with ether, and dried. For a still further purification of the obtained HMG, it is dissolved with distilled water and strained through a column prepared with "Sephadex G 200" suspended in distilled water. The first fractions of the outflow are then collected and then lyophilized. These filtrations on "Sephadex" gel may be effected at a temperature of 4°—5°C.

For the extraction of gonadotropic hormones according to the method of this invention, the menopausal or post-menopausal urine may be collected in glass or vessels made of synthetic plastics materials containing glacial acetic acid in quantities sufficient to ensure that the urine pH is between 4 and 4.5. The purpose of this acidity is to inhibit germ growth.

By the term prewashed kaolin, there is to be understood a kaolin previously treated in the following manner: the kaolin is washed with a normal solution of hydrochloric acid, left to stand, the supernatant liquid is decanted, and the kaolin is washed repeatedly with water to remove the hydrochloric acid.

The diethylaminoethyl-cellulose is previously treated with 0.5N hydrochloric acid, thereafter with 0.5N sodium hydroxide and then with 0.05M phosphate buffer at pH 7.

Preferably there is used as cation exchanger material a "Permutit" cation exchanger material previously treated with 1N ammonium hydroxide, followed by 1N acetic acid, water and finally equilibrated with 0.05M acetate buffer at pH 5.4.

It is understood that in the chromatographic purification of the extract on "Permutit", the ammonium acetate concentration and the degree of ethyl alcohol indicated for HMG elution, are no more than their approximate values, and one can, if one so desired, increase or decrease them, within certain limits.

The procedure of this invention permits the obtaining of gonadotropic hormones with prevalent FSH effect, having the following biological activities:

Total gonadotropic activity (mouse uterus test)
1 mg = 141 mg equivalent of I.R.P.
FSH (Augmentation test in rats)
1 mg = 621 mg equivalent of I.R.P.
LH (Ventral prostate wt. Hypophysectomized rats)
1 mg = 115 mg equivalent of I.R.P.

Total gonadotropic activity: method described by Loraine, J. A. Brown, J. B., J. Endocrinol. 18:77, 1959:

FHS activity: method described by Steelman, S. L., Pohley, Florence M., Endocrinology 53:604, 1953:

LH activity: method described by Loraine, J. A. Brown, J. B., Acta Endocrin. 17:520, 1954.

The results of the biological determinations are calculated according to Gaddum J. H. Pharm. Lond. 6:345, 1953 and Borth, R., Diczafalusy, E., Heinrichs, H. D. Arch, Für Gynäkol. 188:497. 1957.

The "Augmentation test" (Steelman-Pohley) is a method of assaying the FSH activity and is based on the ovarian weight of female immature rats (21 days old) primed with HCG (chorionic gonadotropin). The "ventral prostate weight test on hypophysectomized rats" is a method for assaying the LH activity and is based on the increase of the ventral prostate weight of hypophysectomized 21-day-old rats. The latter two methods are specific for FSH and LH respectively.

The I.R.P. abbreviation means International Reference Preparation of HMG, and is an official standard distributed by the National Institute for Medical Research, Mill Hill, London. The results of tests as expressed in this specification are in terms of I.R.P.

A preferred form of method embodying this invention is exemplified as follows:

EXAMPLE:

A 1083 litre quantity of post-menopausal urine adjusted with glacial acetic acid to pH 4.5, is stirred for about an hour with 5414 g of "Celite" and 54.15 litres of 20% kaolin suspension; the kaolin and "Celite" are left to settle overnight at room temperature. The clear supernatant is discarded and the kaolin plus "Celite" is centrifuged. The centrifugate is washed twice with water at pH 4.5 acidified with acetic acid and then eluted twice with 1M aqueous ammonia in order that the pH of the eluate is between 11 and 11.3. The mixed eluates are brought to pH 8.5 with acetic acid and after separation by centrifugation of the formed precipitate, the obtained clear liquid is acidified to pH 5—5.5, with glacial acetic acid and later 2 Volumes of acetone are added thereto. The precipitate is left to settle overnight and the clear supernatant is discarded. The precipitate is washed with acetone by decantation, then is put into a Buchner filter and washed with 95% ethanol and with ethyl ether, and finally dried over anhydrous calcium chloride under reduced pressure.

The raw kaolin-acetone yield (Fraction A) is 83.88 g.

Fraction A(83.85 g) is extracted twice with 2.516 litres and 1.680 litres of 70% ethanol containing 10% ammonium acetate, each time stirring for about an hour. Two volumes of

absolute ethanol containing 10% ammonium acetate are added to the clear extract obtained from the filtration. The precipitate is left to settle overnight and the clear supernatant is discarded, the precipitate then being collected by centrifugation, washed with 95% ethanol and ethyl ether and dried. 7.527 g of Fraction B are obtained. Fraction B 7.2 g (i.e. 7.2 g taken from Fraction B), are dissolved in 720 ml of 0.05M pH 7 phosphate buffer. The strongly coloured brown solution is agitated for 10 minutes with 72 g of DEAE-cellulose (diethylaminoethyl - cellulose), previously washed with 0.5N hydrochloric acid, then with 0.5N sodium hydroxide, then with distilled water, and finally with 0.05M pH 7 phosphate buffer. The DEAE-cellulose is separated from the liquid by filtration through a Buchner and then washed twice with 720 ml of 0.05M pH 7 phosphate buffer. The mixed filtrates, now clear and of a pale yellow colour, are acidified to pH 5.4 with glacial acetic acid and then cooled to 4°—5°C. The next step of chromatography on "Permutit" is effected as follows: The "Permutit" is put into a 3 x 20 cm column, and washed with 1N ammonia, then with 1N acetic acid, and finally equilibrated with 0.05M pH 5.4 acetate buffer; the chromatography is carried out in a refrigerated room at a temperature between 0° and 6°C., preferably 4°—5°C. The clear liquid obtained after DEAE-cellulose treatment is percolated through the column and the outflow is discarded; the column is washed with 0.05M pH 5.4 acetate buffer until the E280 mμ optical density is near to zero. The proteins adsorbed on "Permutit" are eluted with 40% ethanol containing 10% ammonium acetate, collecting about 350 ml of eluate. This almost colourless liquid is added with stirring to 2.5 volumes of ethanol chilled to 2°—4°C. The precipitate is left to settle overnight at 2°—4°C. The clear natant is discarded and the precipitate collected by centrifugation is washed with absolute ethanol and then with ethyl ether and dried. Fraction C 290 mg is thereby obtained.

In similar manner three other batches were prepared. See Table 1.

TABLE 1

Batch	Urine lt	Fraction A mg/lt	Fraction B mg/lt	Fraction C mg/lt
1	1083	97.4	6.95	0.28
2	16245	97.4	8.72	0.703
3	59877	97.4	10.33	0.595
4	68862	83.0	15.18	0.45

The biological characteristics of the four groups are reported in Table 2.

TABLE 2

BIOLOGICAL ACTIVITY OF FRACTIONS A — C in terms of IRP — HMG

Material assayed	Mouse uterus test					Augmentation test					Ventral prost. wt. hypophysectomized rat test				
	Design	N	λ	R.P.	F. L. P=0.95	Design	N	λ	R.P.	F.L. P=0.95	Design	N	λ	R.P.	F.L. P = 0.95
Fract. A															
1	2+2	20	0.10	0.64	0.50—0.86										
2	2+2	20	0.10	0.64	0.50—0.86										
3	2+2	20	0.10	0.64	0.50—0.86										
4	2+2	20	0.11	0.76	0.58—0.97										
Fract. C															
1	2+2	20	0.12	74.5	54.4—130	2+2	20	0.08	466.8	389—557	2+2	20	0.46	91	31.7—1088
2	2+2	20	0.13	27.7	21.7—45.1	2+2	20	0.1	138	94.2—238	2+2	20	0.28	44.6	24.6—101.4
3	2+2	20	0.14	35	22.8—60.3	2+2	20	0.20	207.6	138—618	2+2	20	0.23	35.2	25.6—58.5
4	2+2	20	0.13	55.5	37 — 80	2+2	20	0.08	264	216—330	2+2	20	0.17	69.6	46.5—111.6

Design= number of level doses used of standard and unknown material assayed.
N = Number of animals used in the experiment.
λ = precision index.
R.P. = relative potency.
F.L. = confidence limits.

As can be seen from Tables 1 and 2, the biological potency and the recovery of biological activity vary from batch to batch.

It is interesting to note that Fraction C has a high FSH:LH ratio that is Fraction C contains predominantly the FSH factor. Some experiments which we have made, the results of which have not yet been published, have demonstrated that during the chromatography on "Permutit" a loss of LH occurs; it is for this reason that there is a predominant effect of FSH in Fraction C. This point is very important because it has been shown that the FSH effect of human gonadotropin is that which is necessary for ovary stimulation, and, on the other hand, that it can be obtained only either in the way pointed out by this invention, or by extracting it post-mortem from human hypophyses. As LH-acting gonadotropic factor, necessary to the transformation of the follicle into *corpus luteum*, and hence to ovulation, also HCG (chorionic gonadotropin) can be used as it has an action similar or equal to that of ICSH, as extracted either from human hypophyses or from post-menopausal urine.

For a further HMG purification, a filtration on "Sephadex" G 200 140—400 mesh has been carried out.

The Sephadex" G 200 is suspended in distilled water and washed by decantation three or four times, with H_2O, thereby discarding the finer fractions. The Sephadex gel is put into a 107×0.9 cm column. The chromatography is carried out in a refrigerated chamber of 4°—6°C.

The $E280^{m\mu}$ optical density of the outflow is measured with a Beckman DU spectrophotometer. The speed of the outflow is regulated at 1 ml per hour. The HMG is dissolved in H_2O. The filtration on the gel requires about 48 hours. After the $E280^{m\mu}$ optical density has been determined, all the collected fractions are frozen and finally lyophilized.

The following is an example of Purification of Fraction C by utilizing the chromatography on Sephadex G 200.

As starting material was used the HMG purified as step C, namely, after chromatography on the cation exchanger column.

60 mg of Fraction C are dissolved in 1 ml of H_2O, and the solution is put into the chromatographic column. The filtration on the gel is effected by distilled water. The outflow is regulated to 1 ml per hour. When the optical density $E\ 280^{m\mu}$ is above zero, the outflow is collected to sum up 57.4 ml.

The different fractions of the outflow, after the $E280^{m\mu}$ has been determined, are frozen and stored at −25°C before the lyophilization.

The results are represented in the accompanying drawing, which is a plot of the chromatography of fraction C of purified HMG on "Sephadex" G 200. In this plot, the continuous line means the Optical density E 280 mμ; the dotted line means the biological activity (as measured by the mouse uterus test); the pointed line means the biological activity (as measured by the augmentation test in rats). As will be seen, the plot shows only one peak; the dotted line indicates a biological activity recovery of 96%, and the pointed line a biological activity recovery of 88%. The overall protein recovery is of 100% (cf.infra).

In previous experiments we ascertained that by using HMG chromatography on "Sephadex" G 75 and DEAE-Sephadex, most of the biological activity was concentrated in the first fractions of the outflow. For this reason, we mixed Fractions 1 to 10 (31.6 ml of the outflow), Fractions 11 to 12 (16.9 ml of outflow), Fractions 13 to 15 (8.9 ml of outflow), and these mixed fractions were lyophilized.

Fraction 1—10 after lyophilization yields 17.5 mg of protein; Fractions 11—12 after lyophilization yields 39.3 mg of protein; Fraction 13—15 after lyophilization yields 3.2 mg of protein.

The biological activity of these fractions is reported in Table 3.

TABLE 3

BIOLOGICAL ACTIVITY OF FRACTIONS OBTAINED WITH CHROMATOGRAPHY ON SEPHADEX G 200 in terms of IRP — HMG

Tested materials	Mouse uterus test					Augmentation test					Ventral prost. wt. hypophysectomized rat test.				
	Design	N	λ	R.P.	F L P = 0.95	Design	N	λ	R.P.	F L P = 0.95	Design	N	λ	R.P.	F L P = 0.95
Starting material purified HMG	2+2	20	0.13	55.5	37 —80	2+2	20	0.08	264	216—330	2+2	20	0.17	69.6	46.5—111.6
Fract. 1—10	2+2	20	0.1	140.8	100—184	2+2	20	0.09	621	454—868	2+2	20	0.28	115.6	69.6—134
Fract.11—12	2+2	20	0.13	18.7	12.3—30	2+2	20	0.12	78	60.6—110.4	2+2	20	0.18	30	14—60.3
Fract.13—15	Inactive at dose levels used.														

For definitions of N, λ, R.P., F.L., refer to Table 2.

As shown in Table 3, the potency of Fraction 1—10, either by the mouse uterus test, or by the augmentation test, is about 2.5 times higher than that of the starting material.

The overall recovery (Fraction 1—10 and Fraction 11—12) of the biological activity is equal to 96%, as referred to the activity of the starting material.

74% of the biological activity of the starting material is concentrated in the first 31.6 ml of the outflow; whereas only 22% of the biological activity is found in Fraction 11—12, while Fraction 13—15 is completely inactive.

The characterization of the protein component of Fraction 1—10 as obtained by chromatography on "Sephadex" G200 as described in the cited example, was obtained by means of immunoelectrophoresis according to the method described by Lunenfeld B. Isersky G. Shelesnyak M. C., J. Clin. Endocrinology & Metab. 22:55, 1962.

On the plate used for the immunoelectrophoresis, besides Fraction 1—10, also normal human serum and a less pure HMG serum having a gonadotropic total potency of 16 mg equivalent of I.R.P. were placed, in order to compare the electrophoretic mobility of the various protein fractions. The anti - HMG serums used were as follows: first, rabbit anti-serum, against total human serum protein; second, rabbit anti-serum against less purified HMG. The following are the results of the immunoelectrophoretic analyses.

Fraction 1—10 with the anti-serum of total human serum protein presents two precipitin lines in the albumin region, but the centre of these lines does not correspond to that of albumin. There is a third hardly visible line, which perhaps coincides with the albumin line. Then there are two lines in the region of α_2-, and one line in the region of β-globulins.

The same Fraction 1—10 with the anti-HMG serum gives two clear lines whose centres are in the region between β- and α-, and in one region β and finally 1 line in region α_1, whereas the less pure HMG. gives 8 precipitin lines.

WHAT WE CLAIM IS:—

1. A pituitary human menopausal gonadotropic (HMG) hormone preparation comprising follicular stimulating hormone (FSH) and luteinizing hormone (LH) having a FSH:LH ratio as hereinbefore defined of at least 3:1 and having a total gonadotropic activity, when determined by the mouse uterus test as hereinbefore defined, in excess of 80 mg equivalent International Reference Preparation (IRP) per mg.

2. A preparation as claimed in Claim 1, wherein the said FSH:LH ratio is between 3:1 and 6:1.

3. A preparation as claimed in Claim 1 or Claim 2, having a total gonadotropic activity (mouse uterus test) of above 80 mg equivalent IRP/mg, an FSH activity (Augmentation test in rats) of at least 220 mg equivalent IRP/mg and an LH activity (Ventral prostate test) of not more than 45 mg equivalent/mg.

4. A preparation as claimed in Claim 1 or Claim 2, wherein the gonadotropic activity (mouse uterus test) is in excess of 100 mg equivalent IRP/mg; the FSH activity (Augmentation test in rats) is above 440 mg equivalent/mg and the LH activity is not in excess of 90 mg equivalent/mg.

5. A pharmaceutical preparation containing as active ingredient a physiologically active quantity of a hormone preparation as claimed in any one of Claims 1 to 4 and lactose as an excipient therefor.

6. A pharmaceutical preparation of predominantly FSH activity and less pronounced LH activity, devoid of antigenic properties when injected into humans and adapted to stimulate human ovaries, comprising as active ingredient a hormone preparation as claimed in any one of Claims 1 to 4.

7. Pituitary human menopausal gonadotropic HMG hormone preparations, substantially as hereinbefore described and with reference to the Example.

8. A method for the production of a pituitary gonadotropic hormone preparation containing FSH and LH at a FSH:LH ratio of at least 3:1 as hereinbefore defined which comprises acidifying menopausal or postmenopausal human urine to a pH between 4.0 and 4.5, adding thereto about 0.5% by weight thereof of siliceous or diatomaceous earth and 1% by weight kaolin (which is added as aqueous suspension), agitating this suspension for about 1 hour, separating the said siliceous or diatomaceous earth and kaolin by settling discarding the supernatant liquid, centrifuging the precipitate, washing the centrifugate twice with acidified water at a pH of 4.5, eluting the precipitate twice with 1M aqueous ammonia so as to obtain an eluate of a pH between 11.0 and 11.3, mixing the eluates, adjusting the pH of the eluate to 8.5, separating the formed precipitates, acidifying the clear liquid to a pH of 5.0 to 5.5, adding thereto 2 volumes acetone, maintaining overnight so as to obtain a precipitate, washing the precipitate with acetone, ethanol and diethyl ether, drying the residue under reduced pressure over a drying agent, extracting with 70% aqueous ethanol containing 10% ammonium acetate, adding to the extract absolute ethanol containing 10% ammonium acetate so as to obtain a precipitate, dissolving the precipitate in 0.05 M phosphate buffer of pH 7, stirring the solution with diethylaminoethylcellulose, acidifying the purified liquid to pH 5.4, passing this liquid through a synthetic alumino-silicate gel cation exchanger column, equilibrated with 0.05M acetate buffer of pH 5.4, eluting the column with 40% aqueous ethanol, containing 10% of ammonium acetate, precipitating with 95% ethanol, washing the precipitate with ethanol and ether, and, optionally, dissolving the precipitate in water and passing the solution through a column packed with a cross-linked dextran gel with random ether linkages to the glucose residues in the polysaccharide chains, and collecting the first fractions of effluent.

9. A process as claimed in Claim 8, wherein the acid used for acidification is acetic acid.

10. A process as claimed in Claim 8 or Claim 9, wherein the kaolin is used as a suspension in water containing about 20% by weight kaolin.

11. A process as claimed in any one of Claims 8 to 10, wherein the kaolin is previously washed with normal HCl and then washed with water repeatedly.

12. A method as claimed in any one of Claims 8 to 11, wherein the diethylaminoethyl-cellulose used is previously treated with 0.5M phosphate buffer of pH 7.0.

13. A method as claimed in any one of Claims 8 to 12, wherein there is used a synthetic alumino-silicate cation exchange material of a granular size passing through 56 mesh (per linear inch) and which is retained by a 76 mesh (per linear inch) sieve.

14. A method as claimed in any one of Claims 8 to 13, wherein the said cation exchange material used is previously washed with acetic acid, with water and dried.

15. A method as claimed in any one of Claims 8 to 13, wherein there is passed through the column of cation exchange material previously to the passage of the substance to be chromatographed 1N ammonia, 1N acetic acid and 0.05M acetate buffer of pH 5.4.

16. A method as claimed in any one of Claims 8 to 15, wherein the chromatography on the said cation exchange material is effected at a temperature of between 0°C and 6°C.

17. A method as claimed in any of Claims 8 to 16, wherein there is passed the said 0.05M acetate buffer of pH 5.4 through the

column of the said cation exchange material until the E 280 mμ optical density is nearly zero.

18. A method as claimed in any one of Claims 8 to 17, wherein the product is further purified by passage through a column of the said cross-linked dextran gel.

19. Process as claimed in Claim 18, wherein the passage through the resin column is effected at a temperature between about 0°C and 8°C.

20. A process as claimed in any one of Claims 8 to 19, wherein the purified product is lyophilized.

21. A process for the preparation of pituitary gonadotropic hormone preparations of the type defined in any one of Claims 1 to 7, substantially as hereinbefore described and with reference to the Example.

22. Pituitary HMG hormone preparations, whenever obtained by a process as claimed in any one of Claims 8 to 21.

MEWBURN ELLIS & CO.,
Chartered Patent Agents,
70 & 72 Chancery Lane,
London, W.C.2.
Agents for the Applicants.

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1 SHEET

COMPLETE SPECIFICATION

This drawing is a reproduction of the Original on a reduced scale